Influence of injected pluripotential (EK) cells on haploid and diploid parthenogenetic development

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SUMMARY

A number of pluripotential embryo-derived EK cells were introduced into the blastocoele of haploid and diploid parthenogenetic embryos which were subsequently transferred to suitable recipients. At autopsy on day 10 or 11 of pseudopregnancy 22\% of decidua in the diploid series contained somite-stage embryos while an additional 12\% contained abnormal egg cylinder-like sacs. In the haploid series, 7\% of the decidua contained somite-stage embryos and an additional 5\% contained abnormal 'sacs'. In 'injected' diploid and haploid 'controls' in which the zonae were pierced with an empty injection pipette 3\% and 0\% respectively of decidua in these two series contained somite-stage embryos, while an additional 17\% and 3\% respectively of decidua in these two series contained abnormal sacs. GPI analysis revealed that the EK cells were incorporated into somite-stage conceptuses in only one third of the diploids and in none of the haploid embryos. Although the presence of EK cells considerably increases the chance of normal embryonic development taking place, a detectable contribution from the EK cells into the resulting somite-stage embryo is apparently not necessary. Possible mechanisms allowing successful early post-implantation development to occur in this study are discussed.

INTRODUCTION

Parthenogenetic mouse embryos generally fail to develop beyond the very early post-implantation period when transferred to pseudopregnant recipients, despite the fact that a high proportion are capable of evoking a decidual response (Graham, 1974; Whittingham, 1980; Kaufman, 1981). Two approaches have so far been successfully employed which enable a proportion of both haploid and diploid parthenogenones to develop to early somite stages. Single blastocysts may be transferred to ovariectomized recipients in which they initially enter the 'delayed' state. They are then allowed to implant and subsequently develop following treatment of recipients with appropriate exogenous hormones (Kaufman, Barton & Surani, 1977). Alternatively, several 8-cell embryos or morulae may be aggregated together to produce a chimaeric individual which then appears to be capable of developing in an 'intact' (non-ovariectomized, non-hormonally treated) recipient (see Kaufman, 1981).
Whatever the reason for the failure of parthenogenetic embryos to complete development, it is not associated with a lack of genetic information for prolonged survival. Thus, for example, teratomas may be induced experimentally following the transfer of blastocyst-stage parthenogenones to ectopic sites (Iles et al. 1975). These teratomas contain a variety of cell types and indicate the degree to which differentiation can occur. Developmentally, cells derived from parthenogenones would appear to be completely normal. When parthenogenetically activated embryos are combined with normal fertilized embryos viable live-born chimaeras are produced (Stevens, Varnum & Eicher, 1977; Surani, Barton & Kaufman, 1977). Some of these chimaeras have proved to be functional germ line mosaics (Stevens, 1978). EC cells isolated from ovarian tumours which have been derived from spontaneously activated oocytes are also capable of normal embryonic development when reintroduced into fertilized embryos (Cronmiller & Mintz, 1978; Illmensee, 1978; Fujii & Martin, 1980). In addition, pluripotential EK cells derived from haploid and diploid parthenogenetically activated oocytes grown entirely in vitro (Kaufman, Robertson, Handyside & Evans, 1983; Robertson, Kaufman, Bradley & Evans, 1983) are capable of participating very extensively in normal development in a chimaeric animal (Robertson, Kaufman & Bradley, unpublished observations).

The reason why parthenogenetically derived cells are capable of developing in a chimaeric association with fertilized cells but not in isolation is not known. One possible hypothesis which could explain these observations, namely that most parthenogenetic blastocysts contain an inadequate number of cells which are destined to form the embryo proper has been proposed (Kaufman, 1981). Consequently, most parthenogenones appear to behave like trophoblast vesicles (Gardner, 1972) which are capable of evoking a decidual response, but fail to give rise to an embryo. In those rare instances in which an embryo does in fact form, it is possible that the number of precursor embryo cells present may have exceeded a certain minimum threshold level (Ansell & Snow, 1975). This hypothesis has obvious limitations in that, for example, it fails to explain why parthenogenones that successfully develop to the limb-bud stage and indeed appear in most cases to be morphologically normal, should die shortly thereafter. Nevertheless, it does appear to provide a partial explanation for the poor developmental capabilities of the non-'delayed' and non-aggregated groups of parthenogenones which are transferred to ‘intact’ recipients.

In order to investigate this topic in more detail, and possibly put the ‘threshold level hypothesis’ to the test, we decided to examine the effect of introducing a small number of fertilized embryo-derived pluripotential EK cells (Evans & Kaufman, 1981) into the blastocoelic cavity of both haploid and diploid parthenogenetic embryos.

The EK cells are essentially identical to embryonal carcinoma (EC) cells (Evans & Kaufman, 1981; Robertson et al. 1983), and similar in size to inner cell mass (ICM) cells. Furthermore, they readily become incorporated into the ICM.
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and, under optimal conditions, are also capable of forming a chimaeric association with the embryonic cells (Robertson et al. 1983) so that they might be expected to colonize both the embryo and/or the extraembryonic membranes.

In the present study, small numbers of EK cells were injected into the blastocoelic cavity of either haploid or diploid parthenogenetic blastocysts, and the developmental potential of the resultant chimaeras examined on the 10th or 11th day of pregnancy, at a time when normal (fertilized) embryos would be expected to be at the early forelimb-bud stage of development and contain at least 20–25 pairs of somites.

MATERIALS AND METHODS

Oocytes from superovulated (C57BL×CBA)F1 hybrid female mice were activated parthenogenetically following a brief exposure to a dilute solution of ethanol in phosphate-buffered saline (PBS; for details of this activation procedure, see Kaufman, 1982). Various haploid and diploid classes of parthenogenone are induced, but only those activated oocytes that developed a single pronucleus following extrusion of the second polar body (uniform haploid class, Kaufman, 1981) were selected following this form of activation for use in this study. Additional groups of oocytes were exposed to a dilute solution of ethanol as described above, but were immediately transferred to medium containing 1 μg/ml Cytochalasin D, and retained in this medium for 4–5 h. This treatment effectively inhibits extrusion of the second polar body, and enables heterozygous diploid embryos (Kaufman, 1981) to be obtained; such embryos develop two pronuclei in the absence of second polar body extrusion.

Both groups of activated oocytes were retained in culture until they reached the expanded blastocyst stage. This stage was usually achieved by about 96–100 h following activation. The haploid and diploid blastocysts thus obtained were treated in one of two ways. They were either assigned to the 'experimental' series, in which case they were injected with 129/Sv/Ev derived EK cells, or to the control series. The EK cell lines used in this study were of two distinct types. The B2B2 and CP1 lines were derived from fertilized 'delayed' blastocysts and both possess a normal euploid XY chromosome complement. The third line used, termed HD14, is derived from a haploid parthenogenetic embryo and has a normal XX chromosome complement. All of these pluripotential stem cell lines were established (for methodology see Evans & Kaufman, 1981) and propagated exclusively on feeder layers of inactivated fibroblasts. The EK cells were prepared for injection by dissociating the small cellular clumps isolated from tissue culture with EDTA/trypsin solution. Injection experiments were performed using cell lines which ranged between 8 to 15 passage generations since their initial isolation in tissue culture. As the lines have not shown distinct characteristics with regard to the experimental results presented here, the findings in each series have therefore been presented as a single group. All three of
these lines have previously been used in our laboratory to produce chimaeric mice when injected into ‘host’ fertilized blastocysts with a considerable degree of success (Robertson et al. 1983; Bradley & Robertson, unpublished results). The experimental embryos in the present study into which EK cells have been introduced will be referred to in the rest of the text as the ‘injected’ blastocysts.

During the injection procedure the blastocyst is immobilized, and all manipulations are carried out in a drop of medium (DMEM+10% Foetal Calf Serum) in a chamber under light paraffin oil at 10°C. Using a Leitz double micromanipulator apparatus, a single sharpened glass injection pipette (internal diameter 15–20 μm) is inserted through the zona pellucida and mural trophoblast layer into the blastocoelic cavity, and a small number of EK cells (generally 5–10) released into this location. Haploid and diploid control groups were established which contained blastocysts which had not at any time been exposed to EK cells. In these control series, the zonas of both haploid and diploid blastocysts were pierced with a glass needle to simulate the injection procedure referred to above, and to facilitate the ‘hatching’ process which is sometimes impaired in parthenogenones (see Discussion).

Both classes of embryos were allowed to re-expand in culture, and were subsequently transferred to ‘intact’ (i.e. non-ovariectomized) recipients (F1 hybrid and CFLP strains) on the 3rd day of pseudopregnancy following mating to F1 hybrid male mice of proven sterility (day of finding vasectomized plug referred to as the first day of pseudopregnancy).

The recipients were autopsied in the morning of the 10th day, or occasionally on the 11th day, of pseudopregnancy, when fertilized embryos would be expected to have 15–20 or over 25 pairs of somites, respectively. The total number of implants present and their contents were recorded. All of the embryonic material in the experimental series, and most of the material in the control series was analysed biochemically. Several grossly abnormal egg-cylinder-like structures, however, from the control series were examined histologically to investigate their morphology.

The larger embryos that had ‘turned’ to adopt the ‘foetal’ position were isolated from their yolk sacs, and the embryonic and extraembryonic samples were analysed separately by one-dimensional gel electrophoresis to determine their respective glucose phosphate isomerase (GPI) allozyme type(s). In the case of the smaller somite-containing ‘unturned’ embryos and the morphologically disorganized ‘egg-cylinder-like’ embryos, the entire conceptus including its membranes was examined as a single unit. This form of analysis enabled the origin of their component parts (whether parthenogenetic embryo- or EK-derived, or of mixed origin) to be determined. This was feasible because the parthenogenones resulted from the activation of F1 hybrid oocytes that were homozygous for the Gpi-1b allozyme, whereas the EK cells were of 129/Sv//Ev origin and homozygous for the Gpi-1a allozyme of glucose phosphate isomerase.
Fig. 1. Examples of somite-containing embryos resulting from the injection of pluripotential EK cells into the blastocoelic cavity of haploid and diploid parthenogenetic blastocysts. All the embryos shown here appeared to be morphologically normal and healthy at the time of their isolation. A. headfold-stage embryo with 7–8 pairs of somites present contained within its yolk sac, from the haploid injected series. B. partially ‘turned’ embryo with about 15 pairs of somites present, from the haploid injected series. C. partially ‘turned’ embryo with about 15–16 pairs of somites present, from the diploid injected series. D. early ‘turned’ embryo with about 20 pairs of somites present, from the diploid injected series.
RESULTS

i) Developmental potential of 'injected' versus 'control' embryos

In the present study, a total of 256 out of 335 (76.4%) of the haploid and diploid 'injected' and 'control' blastocysts successfully implanted (these figures relate to females in which 1 or more decidua were present, and therefore excludes 'recipient failures'). This is similar to the implantation rates achieved in earlier studies in which parthenogenetic blastocysts were transferred to pseudopregnant recipients (see Kaufman & Gardner, 1974; Kaufman et al. 1977; Kaufman, 1978).

Almost 22% of the decidual swellings examined in the diploid 'injected' series contained somite-stage embryos, as did 7.0% of the haploid 'injected' series (see Fig. 1). In addition, morphologically abnormal egg-cylinder-stage or possibly slightly more advanced embryos were also observed in these two 'injected' series. No detailed histological findings are available for this group as all embryos were analysed to determine their GPI content. In the diploid controls, examined on the 11th day, two somite-containing embryos, one with ten pairs of somites and the other with 16–18 pairs of somites were observed. In addition, a number of morphologically abnormal embryos were observed (see Fig. 2). The abnormal embryos proved to be particularly interesting, ranging from a single approximately 18–20 somite-stage embryo with a small mass of tissue which connected the head to the tail region (see Fig. 2C–F), to egg-cylinder-like sacs.

Table 1. Implantation rate and development achieved by parthenogenetic blastocysts injected with EK cells, and uninjected haploid and diploid controls

<table>
<thead>
<tr>
<th>Ploidy</th>
<th>Groups</th>
<th>Embryos transferred</th>
<th>Implants (%)</th>
<th>Abnormal egg cylinder-stage embryos (%)*</th>
<th>Somite-stage embryos</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diploid</td>
<td>'Injected'</td>
<td>138</td>
<td>101 (73-2)</td>
<td>12 (11-9)</td>
<td>22 (21-8)†</td>
</tr>
<tr>
<td>Diploid</td>
<td>Control</td>
<td>83</td>
<td>65 (78-3)</td>
<td>11 (16-9)</td>
<td>2 (3-1)†</td>
</tr>
<tr>
<td>Haploid</td>
<td>'Injected'</td>
<td>72</td>
<td>57 (79-2)</td>
<td>3 (5-3)</td>
<td>4 (7-0)</td>
</tr>
<tr>
<td>Haploid</td>
<td>Control</td>
<td>42</td>
<td>33 (78-6)</td>
<td>1 (3-0)</td>
<td>0 (−)</td>
</tr>
</tbody>
</table>

* percent of implanted embryos.
† $\chi^2$ analysis, $p > 0.01$

Fig. 2. Representative histological sections through two morphologically abnormal conceptuses from the diploid control series. The material has been stained with haematoxylin and eosin. A, B. two sections through an abnormal egg cylinder-like sac containing healthy, but disorganized, tissues. C–F. intermittent serial sections through the cephalic region of an abnormal embryo with about 18–20 pairs of somites present. A small solid mass arises in the cephalic region (C, arrowed) overlying the forebrain (f). This structure becomes canalized (D, arrowed) and the tube thus formed eventually fuses with the neural canal in the tail region (E, F, arrows).
which contained very disorganized and unrecognizable arrays of embryonic tissue (see Fig. 2A, B). In the haploid controls, a single morphologically abnormal egg cylinder stage embryo was observed. A summary of these findings is presented in Table 1.

As far as it was possible to judge on gross inspection, all of the other somite-containing embryos appeared to be morphologically normal with beating hearts and (in the more advanced embryos) a yolk-sac circulation.

**ii) GPI analysis of injected conceptuses**

In the two experimental series reported here, almost three quarters of the somite-stage embryos of diploid origin and all those of haploid origin, appeared to contain cells of Gpi-1b type only. Full details of the GPI results are given in Tables 2 and 3. Interestingly, all but one of the embryos which showed evidence of chimaerism were in the more advanced group, with at least 20–25 pairs of somites present. However, about two thirds of the most advanced embryos did not appear to have any detectable EK contribution. Similarly, there was no apparent evidence of any preferential embryo or yolk sac colonization.

### Table 2. Incidence of chimaerism in parthenogenetic blastocysts injected with EK cells

<table>
<thead>
<tr>
<th>Ploidy</th>
<th>Stage of autopsy</th>
<th>Abnormal egg cylinder-stage embryos</th>
<th>Somite-stage embryos</th>
<th>Pairs of somites present</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>2–8</td>
<td>9–12</td>
</tr>
<tr>
<td>Diploid</td>
<td>11th day</td>
<td>12† (2)*</td>
<td>2</td>
<td>2 (1)*</td>
</tr>
<tr>
<td>Haploid</td>
<td>10th day</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

* Numbers in parentheses indicate embryos with EK cell chimaerism, as revealed by GPI analysis.
† No result was obtained with five of these embryos.

### Table 3. Approximate extent of EK cell contribution in diploid chimaeric embryos and their yolk sacs

<table>
<thead>
<tr>
<th>Embryo</th>
<th>Approximate no. of somites present</th>
<th>EK contribution (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Embryo</td>
</tr>
<tr>
<td>1</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>20</td>
<td>5</td>
</tr>
<tr>
<td>3</td>
<td>25</td>
<td>65</td>
</tr>
<tr>
<td>4</td>
<td>25</td>
<td>10</td>
</tr>
<tr>
<td>5</td>
<td>12</td>
<td>30*</td>
</tr>
</tbody>
</table>

* Embryo and its yolk sac were analysed as a single sample.
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The GPI status of the morphologically abnormal egg cylinder-like sacs proved to be technically rather more difficult to determine, presumably because of the very small volume of tissue generally present in these conceptuses (for details of results see Table 2).

DISCUSSION

Several of the findings in this study were unexpected, and consequently raise issues which justify detailed consideration. Firstly, the relatively high incidence of somite-containing embryos in both the haploid and diploid 'injected' series was surprising, as previous studies had indicated that unless parthenogenones are induced to enter a period of quiescence (i.e. the 'delayed' state) or aggregated together at the 8-cell or morula stage before implantation, the chances of obtaining development beyond the early egg cylinder stage are slight (for discussion, see Kaufman et al. 1977; Kaufman, 1981). Indeed, in the most comprehensive study available, in which embryos were activated and allowed to progress in vivo, the incidence of healthy development to the egg cylinder or early somite stage was 2-3 % when females were autopsied on the 9th or 10th day following electrical stimulation, and the few embryos recovered were retarded in development by approximately 1 day (Tarkowski, Witkowska & Nowicka, 1970). In another series in which in vitro activated eggs were transferred to appropriate recipients, no embryonic development was observed when autopsies were carried out on the 6th or 7th day of pseudopregnancy and the contents of over 130 decidual swellings examined (Kaufman & Gardner, 1974).

In the present study, 21-8 % and 7-0 % of the blastocysts that implanted in the diploid and haploid 'injected' series, respectively, progressed to somite-containing stages of development. In the diploid control series 3-1 % of the blastocysts that implanted developed to this extent, while in the haploid control series no such advanced embryos were observed. Even the relatively small number of somite-containing embryos present in the diploid control series was unexpected, as were the morphologically abnormal conceptuses which were mostly at the egg-cylinder-stage, as previous experience with non-'delayed' material suggested that embryonic losses almost invariably occurred during the very early post-implantation period. It was therefore all the more surprising to find that the stage of development achieved by many of the somite-containing embryos in the two experimental series was similar to that of fertilized embryos isolated from recipients at a comparable stage of gestation. As far as it was possible to judge on gross inspection, virtually all of the somite-containing embryos appeared to be healthy and morphologically completely normal (see Fig. 1). Those embryos with more than 6–8 pairs of somites present all had a beating heart at the time of their isolation.

One possible explanation for the modest incidence of embryonic development (both normal and disorganized) in the control series was that puncturing the zona
pellucida with a needle facilitated the ‘hatching’ process in these groups. Previous observations on embryos cultured \textit{in vitro} (M. H. Kaufman, unpub.) have repeatedly demonstrated that the ‘hatching’ process may be considerably impaired in parthenogenones compared to fertilized embryos. Whereas most expanded blastocysts in the latter group might reasonably be expected to ‘hatch’ in serum-containing medium, far fewer parthenogenones (diploids > haploids) appear to be capable of doing so. If any significant delay in ‘hatching’ occurs \textit{in vivo}, this would almost certainly exaggerate any possible degree of asynchrony present between these embryos and the uterus, and consequently decrease the chance of normal embryonic development occurring. While the eventual exposure of the trophoblast cells to the uterine epithelium would normally be capable of inducing a decidual response, any delay in the appearance of the latter would almost certainly be detrimental to embryonic development. However, the situation in the ‘injected’ group is rather different. It is possible that in these embryos, the enhanced development potential observed was related to the fact that the EK cells rapidly become incorporated into the ICM component of the blastocyst. We believe that the introduction of these cells probably increases its cellular pool, possibly beyond a hypothetical minimum threshold level necessary for embryonic development beyond the early post-implantation period to take place.

It is possible that in those injected blastocysts in this study that implanted but did not form embryos, either insufficient EK cells were injected or, more likely, became incorporated into the ICM, so that the combined EK and ICM cell population was insufficient to enable successful early post-implantation development to take place.

The findings from GPI analysis that the EK cells did not become incorporated into the embryos to form chimaeric individuals in the haploid, and in only one third of the diploid conceptuses was also unexpected, as we have demonstrated that identical EK cells injected into fertilized blastocysts readily gave rise to live-born overtly chimaeric mice (authors, and R. L. Gardner, unpub.). It is unclear whether the injected but apparently non-chimaeric parthenogenetic embryos possessed a low (<5\%), and therefore undetectable EK contribution. The absence of a substantial EK component in two thirds of the somite-stage embryos was all the more surprising because it had been assumed that the haploid parthenogenones in particular would probably act as a ‘passive’ carrier for the EK cells, allowing them to overcome the ‘implantation barrier’ and then take over to form the major component of the embryo. Indeed, only a single ‘injected’ diploid embryo has shown an EK contribution in excess of 50\%.

It is possible that the difference in response observed between the present injection experiments and earlier studies in which similar cells were injected into fertilized blastocysts may reflect subtle differences in the properties and morphology of the ICMs between these two groups. It is relevant to recall that the ICMs of expanded parthenogenetic blastocysts when isolated by immunosurgery
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and subsequently allowed to develop in vitro are still capable of producing typical trophectoderm giant cells (Kaufman, 1981) at a time when similarly isolated ICM cells of fertilized origin would not be capable of doing so (see Handyside, 1978; Spindle, 1978; Rossant & Lis, 1979). The latter property may be related to the relatively smaller cell population present in the ICMs of parthenogenones compared to fertilized blastocysts (Kaufman, 1978), or may reflect the fact that in parthenogenones, at a comparable stage of morphogenesis, fewer ICM cells are irreversibly committed to form embryonic derivatives. Alternatively, primitive endoderm formation may occur precociously in the parthenogenetic group, and thereby form a barrier which effectively excludes the injected EK cells from forming a significant part of the developing embryo. It is possible therefore that a substantial proportion of the injected EK cells may be relegated to an endoderm lineage, or be excluded completely from taking part in subsequent development.

It seems to us that a more detailed analysis of the fate of the EK cell population during the early post-implantation period in 'injected' parthenogenetic and fertilized blastocysts (using appropriately 'labelled' EK cells) might help to clarify some of these issues. This approach would almost certainly shed light 1) on the transformation of teratocarcinoma cells into normal embryonic cells and tissues, and 2) on the behavioural differences between parthenogenetic and fertilized embryos during this period.

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REFERENCES


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